The mitochondria-targeted antioxidant Mitoquinone protects against cold storage injury of renal tubular cells and rat kidneys

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ABSTRACT

The majority of kidneys utilized for transplantation are obtained from deceased donors. These kidneys must undergo cold preservation/storage prior to transplantation in order to preserve tissue quality and to allow time for recipient selection and transport. However, cold storage (CS) can result in tissue injury, kidney discardment, or long term renal dysfunction following transplantation. We have previously determined mitochondrial superoxide and other downstream oxidants to be important signaling molecules that contribute to CS plus rewarming (RW) injury of rat renal proximal tubular cells. Thus, this study’s purpose was to determine if adding Mitoquinone (MitoQ), a mitochondria-targeted antioxidant, to University of Wisconsin (UW) preservation solution could offer protection against CS injury. CS was initiated by placing renal cells or isolated rat kidneys in UW solution alone (4 h at 4°C), UW solution containing MitoQ, or its control compound, Decyltriphenylphosphonium Bromide (DecylTPP) (1 µM—*in vitro*; 100 µM—*ex vivo*). Oxidant production, mitochondrial function, cell viability, and alterations in renal morphology were assessed following CS exposure. CS induced a 2 to 3-fold increase in mitochondrial superoxide generation and tyrosine nitration, partial inactivation of mitochondrial complexes, and a significant increase in cell death and/or renal damage. MitoQ treatment decreased oxidant production ~2-fold, completely prevented mitochondrial dysfunction, and significantly improved cell viability and/or renal morphology, while DecylTPP treatment did not offer any protection. These findings implicate that MitoQ could potentially be of therapeutic use to reduce organ preservation damage, kidney discardment, and/or possibly improve renal function following transplantation.
INTRODUCTION

Deceased organ donors have provided a substantial number of kidneys for patients suffering from end stage renal disease who require transplantation. These kidneys must undergo cold preservation prior to transplantation. The preferred method of organ preservation in the United States is cold storage (CS) and is used in approximately 80% of transplantation cases (Maathuis et al., 2007; Moers et al., 2009). CS slows down metabolic reactions to preserve organ quality while allowing time for recipient selection and transport. Although this procedure is extremely valuable, CS has been shown to cause vasoconstriction, tubular and endothelial injury, and cell death (Salahudeen, 2004; Salahudeen et al., 2001) which can result in kidney discardment. Based on the 2009 Organ Procurement and Transplantation Network/Scientific Registry of Transplant Recipients Annual Report, 16% of kidneys recovered from potential deceased donors were discarded due to cold ischemia times, biopsy findings, or the inability to locate a recipient (Klein et al., 2010). Kidneys that are transplanted following CS, compared to kidneys from living donors, can lead to delayed graft function, chronic allograft nephropathy, graft loss, and/or increased medical cost (Ojo et al., 1997; Schwarz et al., 2005; Wight et al., 2003). Because of these CS outcomes, it is imperative to determine additional strategies to enhance the quality of deceased donated kidneys during preservation.

Many research groups have made significant advances in this area by testing a range of compounds as additives to preservation solutions to improve cellular or tissue function during CS or following transplantation. Recent reports suggest that the addition of bioflavonoids and trophic factor supplementation to preservation solutions should be
explored further because these compounds prevented lipid peroxidation, mitochondrial dysfunction, and loss of cell viability during CS of porcine and canine renal tubular cells (Ahlenstiel et al., 2006; Kwon et al., 2007). The addition of polyethylene glycol (PEG) and trimetazidine to preservation solutions reduced interstitial and peritubular inflammation, infiltration, and renal dysfunction of pig kidneys following cold ischemia/reperfusion (I/R) (Faure et al., 2002; Hauet et al., 2000). The addition of the antioxidant, deferoxamine to the University of Wisconsin (UW) preservation solution has been shown to improve glomerular filtration rate and to decrease cell death in a syngeneic rat kidney transplant model (Huang et al., 2003). Despite these efforts and highly regarded findings, only PEG has been reported to improve kidney preservation in a preliminary clinical study (Codas et al., 2009).

The aim of the present study was to determine if adding Mitoquinone (MitoQ), a mitochondria-targeted antioxidant, to UW preservation solution could ameliorate early CS (4 h) injury using rat renal proximal tubular cells and isolated rat kidneys. MitoQ is comprised of a ubiquinone moiety covalently linked to an aliphatic ten-carbon chain with a triphenylphosphonium cation (Kelso et al., 2001). Once localized to the mitochondria, it is reduced to the active antioxidant ubiquinol by complex II of the electron transport chain. To prevent oxidative damage, it is oxidized to ubiquinone, which is then re-reduced by complex II (James et al., 2005). MitoQ has been shown to be beneficial against oxidative stress, mitochondrial dysfunction, and cell death in cellular and animal models of sepsis, cardiac I/R, and cardiac hypertrophy (Lowes et al., 2008; Adlam et al., 2005; Graham et al., 2009). Additionally, MitoQ has been tested in two Phase II clinical
trials were it was shown to reduce liver damage in patients with chronic hepatitis C virus (Gane et al., 2010; Snow et al., 2010).

MitoQ was selected for this study because it is directly targeted to the mitochondria where we believe the initiating events of CS injury occurs. We recently reported mitochondrial reactive oxygen species (ROS; superoxide, nitric oxide, and peroxynitrite) to be major contributors to mitochondrial dysfunction and oxidant production during 24 h CS and 6 h rewarming (RW) of rat renal proximal tubular cells (Mitchell et al., 2010). Consequently, this appears to be the first report evaluating the benefits of adding a mitochondria-targeted antioxidant to an organ preservation solution. Additionally, MitoQ's control compound Decyltriphenylphosphonium Bromide (DecylTPP) which has a similar chemical structure as MitoQ without the antioxidant ubiquinol moiety was included in this study to determine if the effects of MitoQ were due to its anti-oxidative properties. Our results reveal that adding MitoQ to UW preservation solution provides significant protection against oxidative stress, mitochondrial dysfunction, cell death and renal injury during CS of rat renal proximal tubular cells and rat kidneys.
METHODS

**Chemicals and Reagents:** MitoQ mesylate and DecylTPP bromide were kindly provided by Professor Robin Smith (University of Otago, Dunedin, New Zealand) and Dr. Michael P. Murphy (Medical Research Council Mitochondrial Biology Unit, Cambridge, United Kingdom) (Figure 1A). For the *in vitro* experiments pure MitoQ mesylate and DecylTPP bromide were used, while MitoQ mesylate bound to Cyclodextrin and DecylTPP bromide were used for the *ex vivo* studies. MitoQ mesylate was bound to Cyclodextrin to make MitoQ more stable and easier to handle. Peroxynitrite was supplied by Dr. John Crow’s laboratory (University of Arkansas for Medical Sciences, Little Rock, AR, USA).

**Cold Storage in vitro Model:** Normal rat kidney proximal tubular cells (NRK-52E; No. CRL-1571 American Type Culture Collection, Manassas, VA, USA) were maintained in six-well, 100-mm, or 150-mm size plates in a humidified incubator gassed with 5% CO₂, 95% air at 37°C in DMEM (Invitrogen, Eugene, OR, USA) containing 5% fetal calf serum (FCS). Cells were grown to 60% confluency, and divided into four treatment groups: (1) Untreated (Untx), (2) Cold Storage (CS), (3) CS+MitoQ, (4) CS+DecylTPP. Untreated cells remained at 37°C in DMEM containing 5% FCS (Group 1). CS was initiated by washing cells with cold PBS (Invitrogen, Eugene, OR, USA) twice and storing them in University of Wisconsin/Viaspan (UW) solution alone (4 h at 4°C) (Group 2), CS+MitoQ (1µM) (Group 3), or CS+DecylTPP (1µM) (Group 4). In separate experiments, cells were exposed to CS plus rewarming (CS.RW) by replacing UW solution alone or UW containing MitoQ or DecylTPP with DMEM containing 5% FCS overnight (18 h at 37°C).
Cold Storage ex vivo Model: All animals were treated according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee guidelines. Male Fischer 344 inbred rats (Charles Rivers Laboratories, Inc., Wilmington, MA, USA) weighing between 250 to 300 grams were anesthetized with ethrane, followed by shaving and prepping with betadine. A 2-mL bolus of 0.9\% (w/v) NaCl was administered intravenously, and an incision was made 1 cm superior to the symphysis pubis to the tip of the xiphoid process. Bulldog clamps were placed on the aorta and vena cava (proximal and distal to the renal vessels) to prevent blood flow to the kidneys. A 22 gauge surgical needle was used to create a puncture in the rat’s aorta to flush the renal grafts with saline (10 mL per kidney) using a small catheter. Once kidneys started to turn light brown (perfusion), another vent was formed in the vena cava to allow blood flow from the kidneys. Once both kidneys were completely flushed, the right kidney was recovered and served as a control (group 1); while the left kidney was exposed to CS alone for 4 h at 4°C (group 2). In additional experiments, kidneys were flushed with saline through the aorta using a small catheter followed by flushing the right kidney with saline containing MitoQ (100 \mu M) and the left kidney with saline containing DecylTPP (100 \mu M) (10 mL per kidney). Tissues were recovered and stored in CS+MitoQ (100 \mu M; right kidney) or CS+DecylTPP (100 \mu M; left kidney) for 4 h at 4°C (groups 3 and 4 respectively). A thin middle section from all of the kidneys were cut and immediately fixed in 10\% formalin before being embedded in paraffin for sectioning (4 \mu m) and histological evaluation. The remaining portion of the kidneys were quickly frozen in liquid nitrogen and stored in -80°C until needed for biochemistry analyses.
Mitochondrial Superoxide Production: MitoSOX Red (Invitrogen, Eugene, OR, USA) was used to measure mitochondrial superoxide generation during CS. Briefly, cells were preloaded in the dark with MitoSOX red (5 µM; 10 min at 37°C) prior to treatment (groups 1-4). Fluorescence was visualized using a Nikon Eclipse E800 microscope (Melville, NY, USA) with a rhodamine filter using a water immersion objective (60x). Fluorescent images of NRK cells were captured with equal exposure times and quantified by averaging the mean intensity fluorescence of 5 random cells in three different fields using Nikon Nis Elements software. In separate experiments, cells were grown on coverslips, preloaded with MitoSOX Red prior to treatment (groups 1-4) and evaluated for mitochondrial superoxide generation using a Hitachi F-2500 spectrofluorometer (Hitachi, Tokyo, Japan) equipped with a coverslip holder using 2 different excitation wavelengths: 396 nm and 510 nm with the emission measured at 580 nm as previously described (Munusamy and MacMillan-Crow, 2009). Additionally, rat kidneys were monitored for mitochondrial superoxide generation by flushing kidneys with saline through the aorta using a small catheter as described in the cold storage ex vivo model section above, followed by saline containing MitoSOX Red (5 µM; 10 mL/kidney) prior to treatment (groups 1-4). Paraffin sections were analyzed using the rhodamine filter on the Nikon Eclipse 800 microscope (20x).

Nitrotyrosine Immunocytochemistry and Immunohistochemistry: NRK cells (groups 1-4) were assessed for nitrotyrosine formation as previously described (Mitchell et al., 2010). Briefly, cells were immunostained with nitrotyrosine polyclonal antibody (1:200; Millipore, Billerica, MA, USA), Alexa-594 conjugated antibody (1:1000; Invitrogen, Eugene, OR, USA) and DAPI (1:100; Invitrogen, Eugene, OR, USA) for
nuclear counterstaining. Positive controls were cells treated with peroxynitrite (0.8 mM) in PBS for 5 min at RT. Negative controls were cells treated with peroxynitrite but the antibody binding specificity was confirmed by blocking the nitrotyrosine antibody with excess 3-nitrotyrosine (10 mM; Sigma-Aldrich, St. Louis, MO, USA). Nitrotyrosine staining was evaluated and captured with equal exposure times using a Nikon Eclipse 800 microscope (40x oil). Images were quantified by averaging the mean intensity fluorescence of 5 random cells in three different fields using Nikon Nis Elements software. Renal tissue sections were assessed for the presence of nitrotyrosine protein adducts by nitrotyrosine immunohistochemistry. Tissue sections were deparaffinized and rehydrated, followed by antigen retrieval (20 min incubation at 95°C with 10 mM sodium citrate buffer, pH 6.0; Sigma-Aldrich, St. Louis, MO, USA). Sections were blocked with peroxidase suppressor (Thermo Scientific, Rockford, IL, USA), DAKO protein block (Dako North America, Inc., Carpinteria, CA, USA) and incubated overnight at 4°C with the polyclonal rabbit anti-nitrotyrosine antibody (1:3000). Sections were developed using the DAKO LSAB+System-HRP kit (Dako North America, Inc., Carpinteria, CA, USA) followed by Mayer’s Hematoxylin counterstaining (Electron Microscopy Sciences, Hatfield, PA, USA), dehydration, and mounting with Cytoseal-60 (Electron Microscopy Sciences, Hatfield, PA, USA). The specificity of nitrotyrosine antibody binding in the renal tissue was confirmed by blocking the antibody with 3-nitrotyrosine (10 mM).

**Mitochondrial Respiratory Complex Activity:** NRK cell and rat kidney mitochondria were isolated by centrifugation on a sucrose density gradient. The activity of mitochondrial respiratory complexes I, II, III or IV were assayed spectrophotometrically.
using 100 µg of mitochondrial protein at 30°C as previously described (Birch-Machin and Turnbull, 2001; Saba et al., 2008).

**Morphological Assessments:** Renal sections were assessed for tissue injury using the Periodic Acid-Schiff (PAS) reaction. Tissues were deparaffinized and rehydrated, followed by oxidation with 0.5% periodic acid solution (Sigma-Aldrich, St. Louis, MO, USA) for 5 min at RT. Sections were rinsed with distilled water and incubated with Schiff reagent (Sigma-Aldrich, St. Louis, MO, USA) for 15 min at RT. Subsequently, sections were washed with tap water, counterstained with Mayer’s hematoxylin, dehydrated, and mounted with Cytoseal-60. Morphological evaluation of tubular dilation, brush border loss, cellular debris/tubular cast formation were performed blindly with light microscopy and assessed semi-quantitatively according to the following scoring system: 0-no changes, 1-mild changes, 2-moderate changes, and 3-severe changes.

**Cell Death Measurement:** Cell cytotoxicity was determined using the LDH-Cytotoxicity Assay Kit II (Biovision Research Products, Mountain View, CA, USA) according to the manufacturer’s instructions. Absorbance was measured at 450 nm using a SpectraMax 190 microplate reader and SpectraMax software (Molecular Devices, Sunnyvale, CA, USA). Renal cell death was determined using TUNEL staining. Kidney sections were deparaffinized, rehydrated, and exposed to antigen retrieval (20 min incubation at 95°C with 10 mM sodium citrate buffer, pH 6.0) followed by washing with distilled water and quenching of endogenous peroxidase. Sections were immersed in TdT labeling buffer and then incubated in a humidified chamber with the labeling reaction mix from the TACS® 2 TdT-DAB *In situ* Apoptosis Detection kit (Trevigen, Inc., Gaithersburg, MD, USA) for 1 h at RT. The reaction was stopped with TdT stop buffer and slides were
subsequently washed with PBS. Sections were incubated for 10 min with Streptavidin-HRP followed by development with DAB solution, counterstaining with Mayer’s Hematoxylin, dehydration, and mounting with Cytoseal-60. The amount of cell death was calculated by averaging the number of cells positive for TUNEL (brown staining) in ten different fields (cortex and medulla) (20x). All images were taken at equal exposure times (40x oil).

**Statistical Analysis:** Results are presented as mean ± standard error of the mean (SEM). Means were obtained from three (*in vitro*) or five (*ex vivo*) independent experiments. One way analysis of variance (ANOVA) was used to compare the mean values among the untreated/controls and treatment groups, followed by Tukey’s test to compare differences in mean between two groups at 95% level of confidence using the Origin 6.0 statistical software. Differences with a P value less than 0.05 was considered statistically significant.
RESULTS

Protective effect of MitoQ on mitochondrial superoxide generation during CS.

The optimal dose for MitoQ and DecylTPP treatment were selected from dose response experiments during 4 h CS. For the cellular based studies, 1 µM was chosen from a range of 0.5, 0.75, 1.0, 1.5, and 2 µM; while, the most efficacious dose for the ex vivo studies was 100 µM from a dose response study of 50, 100, and 500 µM (data not shown). The potential protective benefits of MitoQ treatment against CS injury were tested initially using MitoSOX Red, a mitochondrial targeted fluorescent dye that measures mitochondrial superoxide generation. As shown in Figure 2A, NRK cells exposed to CS resulted in a ~2-fold increase in mitochondrial superoxide compared to untreated cells. MitoQ offered significant protection against CS induced mitochondrial superoxide generation; whereas, the control compound DecylTPP did not offer any protection. This was further confirmed using a spectrofluorometric based assay detecting MitoSOX Red fluorescence excitation at 396 and 510 nm, where 396 nm is a specific indicator of mitochondrial superoxide, while 510 nm detects non specific oxidant generation (Robinson et al., 2006) (Figure 2B). In addition, kidneys exposed to CS alone displayed increased mitochondrial superoxide generation compared to control kidneys (Figure 2C). MitoQ treatment markedly decreased mitochondrial superoxide generation, whereas kidneys treated with DecylTPP had comparable levels of mitochondrial superoxide to kidneys exposed to CS alone (Figure 2C).

MitoQ attenuates nitrotyrosine adduct formation during CS.
Immunocytochemistry and immunohistochemistry were used to evaluate nitrotyrosine protein adducts during CS. As shown in Figure 3A, NRK cells exposed to CS alone had a significant increase in nitrotyrosine staining (red fluorescence) compared to untreated cells. MitoQ attenuated nitrotyrosine formation ~2-fold, whereas the control compound DecylTPP, did not decrease CS mediated nitrotyrosine formation (Figure 3A). Peroxynitrite treated cells had intense nitrotyrosine formation (positive control) and was blocked when the nitrotyrosine antibody was preabsorbed with excess 3-nitrotyrosine (negative control; ONOO\(^-\) +Block). Rat kidney immunohistochemistry data was consistent with the \textit{in vitro} study findings regarding the effect of MitoQ and nitrotyrosine. Figure 3B shows an increase in nitrotyrosine (brown staining) in the distal and proximal tubules and to a lesser extent in the glomeruli of CS kidneys compared to control kidneys. Kidneys treated with MitoQ had less nitrotyrosine formation. In contrast, DecylTPP treated kidneys had similar amounts of nitrotyrosine compared to CS kidneys. The specificity of nitrotyrosine staining was also confirmed using antibody preabsorbed with excess 3-nitrotyrosine (CS+Block).

\textit{MitoQ prevents mitochondrial respiratory complex inactivation during CS.}

Mitochondrial respiratory complex activity was evaluated in both the \textit{in vitro} and \textit{ex vivo} renal models to investigate whether 4 h CS alters mitochondrial respiratory function. Complexes I and II were significantly inactivated following CS of NRK cells compared to untreated cells (Figure 4). MitoQ completely prevented Complex I and II inactivation, while DecylTPP had no significant effect on complex activity. Complexes III and IV were not assessed in this study because we have previously shown both complex activities to be unchanged with 24 h CS (Mitchell et al., 2010), therefore
evaluation of these complex activities were not warranted. Consistent with the in vitro findings, CS of rat kidneys led to partial inactivation of Complexes I and II activity and had no effect on Complexes III and IV (Figure 4). MitoQ protected against Complex I and II inactivation of CS kidneys, while DecylTPP did not have any effect.

CS induced renal injury and cell death is decreased with MitoQ treatment.

Periodic Acid-Schiff staining was performed to examine histopathological changes during CS of rat kidneys. Significant widespread tubular damage such as: dilation, brush border loss, and cellular debris/cast formation occurred with CS exposure (Figure 5A). MitoQ improved renal histology significantly while DecylTPP treatment did not reverse renal injury. TUNEL staining, indicated by brown staining of the nuclei, was used as a marker of cell death. As shown in Figure 5B, CS led to a significant increase in cell death (red arrows) compared to control kidneys. MitoQ treatment decreased cell death by ~2-fold compared to CS kidneys, while DecylTPP offered no protective benefits against cell.

MitoQ decreases nitrotyrosine formation and cell death during CS plus Rewarming of NRK cells.

To test whether MitoQ could potentially offer protection against oxidant production and cell death following reperfusion/transplantation, cells were exposed to CS plus RW (CS.RW). As shown in Figure 6A, 4 h CS plus overnight (18 h) RW of NRK cells resulted in significant nitrotyrosine formation. Adding MitoQ during CS, attenuated nitrotyrosine staining significantly during CS.RW whereas, the control compound DecylTPP had no effect. LDH cytotoxicity revealed a significant increase in cell death of
NRK cells exposed to CS.RW compared to untreated cells (Figure 6B). MitoQ decreased cell death approximately 2-fold during CS alone and CS.RW. DecylTPP did not reduce cell death in either treatment groups.
DISCUSSION

In the present study, we have demonstrated that adding the mitochondria-targeted antioxidant, MitoQ, to UW preservation solution decreases oxidant production, prevents mitochondrial dysfunction, and minimizes renal injury and cell death during CS of NRK cells and isolated rat kidneys. MitoQ’s control compound, DecylTPP (the lipophilic cation that targets MitoQ to the mitochondria) offered no protective effect and was included in this study as a negative control and to confirm MitoQ’s anti-oxidative properties. It is important to point out that MitoQ was administered only during CS (4°C) throughout this study. Thus, it is an ideal drug candidate for improving organ preservation because it would not require administration to the deceased donor prior to explantation nor during organ reperfusion of the transplant recipient. During cold preservation of human organs, biopsies that evaluate renal damage are often used to determine whether to transplant or discard a donated kidney. Therefore, MitoQ’s ability to reduce histological evidence of renal injury suggests that MitoQ might lead to fewer kidneys being discarded due to CS mediated damage. Moreover, MitoQ has been proven to be safe and well tolerated in two Phase II clinical trials for Parkinson’s disease and Hepatitis C virus (Snow et al., 2010; Gane et al., 2010), which could expedite its testing in clinical trials for use during organ preservation. In addition, MitoQ would not be administered systemically to patients, so potential drug-induced side effects would be greatly minimized. Results from the present study using markers of oxidative stress, mitochondrial function, and cellular injury provide substantial evidence of the effectiveness of MitoQ against CS-mediated injury and support the notion that
mitochondrial ROS should be an important target in preventing cold preservation injury of donated kidneys.

Previous studies have shown ROS, including superoxide (NADPH oxidase as the source), hydrogen peroxide, and hydroxyl radical to be major contributors to CS injury using human and porcine renal tubular cells (Salahudeen et al., 2000; Karhumaki et al., 2007). Our laboratory recently reported mitochondrial ROS (superoxide, nitric oxide, and peroxynitrite) to be signaling molecules that contribute to mitochondrial dysfunction and cell death of rat renal tubular cells exposed to CS (24 h) plus RW (6 h) (Mitchell et al., 2010). It is important to point out that this previous study measured CS injury at a later time point (24 h) because most donated kidneys are preserved for extended periods. In the present study, CS events were measured at 4 h to determine how early renal and mitochondrial injury occurs and to establish if preventing early CS mediated injury could avert downstream toxic events.

MitoQ’s potential protective effects during CS were initially tested by measuring mitochondrial superoxide generation using MitoSOX Red. CS induced mitochondrial superoxide was significantly reduced with MitoQ treatment; suggesting, MitoQ would be highly effective in reducing downstream oxidant production and injury. Previous reports have shown MitoQ to decrease mitochondrial ROS specifically derived from superoxide in a hyperglycemic endothelial cell model and in fenretinide treated neuroblastoma cell lines (Quijano C. et al., 2007; Cuperus et al., 2010). In the latter study, fenretinide treatment induced apoptosis of neuroblastoma tumors by increasing mitochondrial ROS production. Superoxide is the proximal oxidant that leads to the formation of other deleterious oxidants and can cause additional damage within the cell by entering the
intermembrane space through the mitochondrial transition pore and/or through the voltage dependent anion channel (Turrens, 2003).

Nitric oxide is a free radical that is well known to interact with superoxide to form peroxynitrite. It is generated by nitric oxide synthases (NOS) and is an important signaling molecule in many physiological processes. Few reports have evaluated the effect of cold ischemia alone on NOS protein levels or activity. Desrois et al. reported endothelial NOS protein levels to decrease significantly during cold ischemia and NOS activity to initially increase following 3 h ischemia but significantly declined after 6 h cold ischemia in a heterotopic rat heart transplantation model (Desrois et al., 2005). The authors suggested that cold ischemia induced endothelial dysfunction in the rat heart by decreasing nitric oxide’s ability to exert its protective effects. In our recent manuscript, available nitric oxide levels declined significantly following 24 h CS of NRK cells, which was likely due to nitric oxide interacting with mitochondrial superoxide to form peroxynitrite (Mitchell et al., 2010). The fact that the NOS inhibitor, N\(^5\)-[imino(methylamino)methyl]-L-ornithine citrate (L-NMMA), inhibited nitrotyrosine staining (one marker of peroxynitrite), strongly suggests that nitric oxide was indeed formed, but was quickly converted to peroxynitrite by interacting with superoxide. Additional studies using the CS NRK cell model showed that available nitric oxide levels were also decreased following 4 h CS and MitoQ had no effect (data not shown).

MitoQ’s effectiveness against CS induced mitochondrial superoxide warranted further evaluation of tyrosine nitration during CS of NRK cells and isolated rat kidneys. Formation of nitrotyrosine can adversely affect protein function and additional cellular processes. We have previously determined that cold I/R of rat kidneys resulted in
nitrotyrosine formation (Saba et al., 2008) and based on our current findings we suggest that cold storage alone increases tyrosine nitration. MitoQ was able to markedly reduce nitrotyrosine in both the cellular and isolated kidney models. After detoxifying oxidants, MitoQ can be reduced by the respiratory chain to be repeatedly recycled back to its antioxidant form (James et al., 2005); which, further supports the advantage of adding MitoQ during CS because it is able to continuously scavenge oxidants. Taken together, our data suggest that adding MitoQ to UW solution attenuates oxidative stress during CS by scavenging mitochondrial superoxide.

Increased oxidant production can lead to mitochondrial respiratory complex inactivation, decline in adenosine triphosphate levels, mitochondrial dysfunction, and consequently cell death (Fosslien, 2001). Cold preservation has been shown to impair mitochondrial function in many organ systems (Meng et al., 1991; Salahudeen et al., 2003; Kuznetsov et al., 2004). Our data reveal that CS leads to partial inactivation of mitochondrial complexes I and II in both the cellular and ex vivo kidney models and excitingly was completely prevented with MitoQ treatment. Protecting complex I and II activity is important because these complexes initiate oxidative phosphorylation and can generate additional oxidants once inactivated. MitoQ was recently shown to restore mitochondrial function in a rodent model of cardiac dysfunction induced by sepsis (Supinski et al., 2009). The authors previously determined that cardiac mitochondrial dysfunction in this model was associated with a significant increase in ROS generation. Thus, MitoQ’s ability to restore CS respiratory complex activity could explain the reduction in mitochondrial superoxide generation also observed with MitoQ.
Both cold ischemia mediated oxidant production and mitochondrial dysfunction can lead to irreversible renal injury and cell death that can result in further damage once the organ is transplanted. It has been shown that loss of tubular brush border is a clear indicator of ischemic injury and a sign of impaired proximal tubular function (Venkatachalam et al., 1978) which can ultimately lead to cell death. In the current study, MitoQ protected against CS mediated renal injury by decreasing tubular brush border loss, tubular dilation, and cell death.

Unfortunately, our laboratory does not have the surgical expertise to transplant CS kidneys into a rat recipient to evaluate renal function following transplantation. However, the extent of protection observed with MitoQ during CS alone clearly indicates that renal function would most likely be improved, and as mentioned earlier protection during CS alone could translate in to fewer kidneys being discarded. Future studies, via a collaborative effort, will be performed in a porcine renal model of CS followed by transplantation to address if MitoQ can attenuate injury following transplantation. This larger animal model is more clinically relevant and technically easier for surgical transplantation studies to be executed. Nevertheless, we were able to measure the benefits of MitoQ treatment against nitrotyrosine formation and cell death following CS and subsequent RW of NRK cells, which is a respectable model that simulates what occurs at the cellular level following rewarming/reperfusion (Stec et al., 2007; Waller et al., 2007). Our recent manuscript showed that CS alone (24 h) increased superoxide and nitrotyrosine levels with minimal cell death, while RW NRK cells increased cell death significantly (Mitchell et al., 2010). These data were similar to what we show in the current manuscript at an earlier time point, and importantly how MitoQ treatment
protected against this injury. Collectively, these findings suggest that the mechanisms leading to cell death during CS.RW appear to be initiated during CS alone, and can be modulated with antioxidant treatment.

In summary, this is the first report showing that mitochondrial superoxide increases significantly during early CS and that it contributes to mitochondrial and renal damage. We have identified the mitochondria-targeted antioxidant, MitoQ to significantly protect against CS-mediated oxidative stress, mitochondrial dysfunction, cell death, and renal injury of renal proximal tubular cells and isolated rat kidneys. These findings suggest that infusion of MitoQ to kidneys prior to transplantation may be of therapeutic use to reduce CS damage, improve outcome for transplant recipients, and also increase the numbers of donated organs available for transplant, all of which could lead to a decline in health care costs.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: MacMillan-Crow, Murphy, and Mitchell

Conducted experiments: Mitchell, Rotaru, and Saba

Contributed reagents: Smith and Murphy

Performed data analysis: Mitchell and MacMillan-Crow

Wrote or contributed to the writing of the manuscript: Mitchell, MacMillan-Crow, Murphy, Saba, Rotaru, and Smith
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FOOTNOTES

A portion of this research was previously presented at Experimental Biology 2010 Meeting, Anaheim, CA.
LEGENDS FOR FIGURES

Figure 1: Chemical structures of Mitoquinone (MitoQ) and Decyltriphenylphosphonium Bromide (DecylTPP) (modified from Kelso et al., 2001).

Figure 2: Effect of MitoQ on mitochondrial superoxide generation during cold storage (CS). MitoSOX Red (5 µM for 10 min) was used to assess mitochondrial superoxide levels. (A). Fluorescence microscopic images of (top two panels): untreated (Untx) cells, cells exposed to CS, and (bottom two panels) cells exposed to CS+MitoQ (1 µM) and CS+DecylTPP (1 µM) (60x). Results are representative of three experiments using different cell cultures. MitoSOX Red staining was quantified using the Nis Elements software. Values are expressed as mean ± SEM (n = 3). **P < 0.01 compared to Untx cells; †P < 0.05 compared to CS cells. (B). Fluorescence spectrometry of NRK cells grown on coverslips and tested for fluorescence of MitoSOX Red at wavelengths 396 and 510 nm excitation/580 nm emission. Values are expressed as mean fold changes in arbitrary fluorescence units over Untx cells ± SEM (n=3). **P < 0.01 compared to Untx cells; †P < 0.05 compared to CS cells. (C). Fluorescence photomicrographs of rat kidneys flushed with MitoSOX Red (left to right): Control, CS, CS+MitoQ (100 µM), and CS+DecylTPP (100 µM) (20x). Results are representative of five animal experiments.

Figure 3: MitoQ attenuates nitrotyrosine formation during cold storage (CS). Cells were immunostained with nitrotyrosine polyclonal antibody (1:200), Alexa-594 conjugated antibody (1:1000) and DAPI (1:100) for nuclear counterstaining. Cells positive for nitrotyrosine fluoresce red. (A). Fluorescence microscopic images of (top two panels): untreated (Untx) cells, cells exposed to CS, and (middle two panels) cells exposed to
CS+MitoQ (1 µM) and CS+DecylTPP (1 µM). The images depicted in the bottom two panels represent staining controls: NRK cells treated with peroxynitrite (ONOO⁻; positive control) and NRK cells treated with ONOO⁻ but preincubated with excess 3-nitrotyrosine (ONOO⁻ + Block; negative control) (40x oil). Results are representative of three experiments using different cell cultures. Nitrotyrosine staining was quantified using the Nis Elements software. Values are expressed as mean ± SEM (n = 3). *P < 0.05 or ***P < 0.001 compared to Untx cells; †P < 0.05 compared to CS cells. (B). Photomicrographs of nitrotyrosine immunohistochemistry of rat kidneys (top two panels): Control, CS, and (middle two panels) kidneys exposed to CS+MitoQ (100 µM) and CS+DecylTPP (100 µM) and (bottom panel) CS+Block (CS kidney+10mM 3-nitrotyrosine) (20x). Results are representative of five animal experiments.

**Figure 4:** MitoQ prevents mitochondrial respiratory complex inactivation during cold storage (CS). Individual mitochondrial respiratory complex activities were measured using isolated mitochondria from NRK cells (Graphs A-B) and isolated rat kidney mitochondria (Graphs C-F) exposed to CS, CS+MitoQ, or CS+DecylTPP (1 µM in vitro and 100 µM ex vivo). Values are expressed as percentage mean ± SEM (n = 3 in vitro or 5 ex vivo) of respective controls (set to 100). **P < 0.01 or ***P < 0.001 compared to respective controls. †P < 0.05 compared to CS.

**Figure 5:** Effect of MitoQ on cold storage (CS) induced renal injury and cell death. Representative photomicrographs from five animal experiments of: (A) Periodic Acid-Schiff (PAS) staining of rat kidneys (left to right): Control, CS, CS+MitoQ (100 µM), and CS+DecylTPP (100 µM) (20x). Extensive tubular dilation, brush border loss, and cellular
debris/tubular cast formation were observed and assessed blindly and semi-quantitatively according to the following scoring system: 0-no changes, 1-mild changes, 2-moderate changes, and 3-severe changes. (B). TUNEL staining (brown nuclei; red arrows) of rat kidneys (left to right): Control, CS, CS+MitoQ (100 µM), and CS+DecylTPP (100 µM) (40x). The amount of cell death was calculated by averaging the number of cells positive for TUNEL in ten different fields of the cortex and medulla. Values are expressed as mean ± SEM (n = 5). ***P < 0.001 compared to control. †P < 0.05 compared to CS.

**Figure 6:** MitoQ decreases cold storage plus rewarming (CS.RW) oxidant production and cell death. (A). Fluorescence microscopic images of nitrotyrosine immunocytochemistry (top two panels): untreated (Untx) cells, cells exposed to CS.RW, and (bottom two panels) cells exposed to CS.RW+MitoQ (1 µM) and CS.RW+DecylTPP (1 µM) (40x oil). Results are representative of three experiments using different cell cultures. Nitrotyrosine staining was quantified using the Nis Elements software. Values are expressed as mean ± SEM (n = 3). *P < 0.05 or ***P < 0.001 compared to Untx cells; †P < 0.05 compared to CS.RW cells. (B). Percent cell death was determined using the LDH Cytotoxicity Assay Kit II. Values are expressed as percentage cell death means ±SEM (n=3). **P < 0.01 or ***P < 0.001 compared to untreated cells. †P<0.05 compared to CS cells. ‡P<0.01 compared to CS.RW cells.
Figure 2

A.

B.

C.
Figure 3

A.

B.
Figure 4

**In vitro**

A. Complex I

Ex vivo

C. Complex I

D. Complex II

E. Complex III

F. Complex IV
Figure 5

A.

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B.

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![Graphs](image17)
Figure 6

A.

![Images of cell fluorescence](untx, CS.RW, CS.RW+MitoQ, CS.RW+DecylTPP)

B.

![Bar graph of percent cell death](untx, CS, CS.RW, CS, CS.RW+MitoQ, CS, CS.RW+DecylTPP)